

# Expression, purification and characterization of a recombinant Tat<sub>47-57</sub>-Oct4 fusion protein in *Pichia pastoris*

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Received May 22, 2013; Accepted December 5, 2013

DOI: 10.3892/mmr.2013.1857

**Abstract.** The transcription factor, Oct-4, is involved in the self-renewal of undifferentiated embryonic stem cells, and is also significant in the reprogramming process and in the development of tumors. In the present study, the fusion protein, Tat<sub>47-57</sub>-Oct4, was secreted by the signal peptide of human serum albumin in *Pichia pastoris* under the control of alcohol oxidase promoter 1. The yield of recombinant Tat<sub>47-57</sub>-Oct4 fusion protein was ~210 mg/l. Following pilot-scale fermentation, Tat<sub>47-57</sub>-Oct4 was purified by ammonium sulfate precipitation, Vivaflow 200 ultrafiltration and SP Sepharose fast flow chromatography in order to obtain 95.6% purity. Immunofluorescence analysis validated the ability of Tat<sub>47-57</sub>-Oct4 to cross the cell membrane. The results demonstrated that the experimental procedure developed in the present study could produce large quantities of active Tat<sub>47-57</sub>-Oct4 fusion protein from *P. pastoris*.

## Introduction

The expression of human Oct4, Sox2, c-Myc and Nanog can turn a variety of differentiated cells into induced pluripotent stem cells with embryonic stem cell-like properties (1-3). Oct4 is a member of the POU transcription factor family, and is significant in the pluripotency and self-renewal of embryonic stem cells (4,5). Induced pluripotent stem cell formation can be caused by Oct4 alone in mouse and human neural stem cells (6,7).

Oct4 only exhibits these functions intracellularly (8), whereas the human immunodeficiency virus (HIV) Tat protein can be taken up by cells where it activates viral genome transcription (9). In the late 1990s, the first use of Tat as a delivery agent to introduce proteins into cells *in vitro* (10) and in mice *in vivo* (11) was reported, followed by numerous studies concerning the application of Tat to deliver various proteins into cells in the form of Tat-fusion proteins or Tat-protein

conjugates (12-14). In the present study, fusing the Oct4 gene with Tat<sub>47-57</sub> allowed Oct4 to penetrate the cell membrane.

Oct4 has been produced using *Escherichia coli* (15) or mammalian cells (16). However, these expression systems are limited by low yields, complex manipulations or high culture costs. The methylotrophic yeast, *Pichia pastoris*, has the advantage of eukaryotic and prokaryotic expression systems and has widely been used to express a variety of biologically active proteins in extremely high yields (17-20). In the present study, the Oct4 gene was fused with a human serum albumin (HSA) signal peptide and HIV Tat<sub>47-57</sub>, and then inserted into the pPICZαC vector. The recombinant Tat<sub>47-57</sub>-Oct4 fusion protein was expressed with *P. pastoris* X-33 under the control of alcohol oxidase 1 (AOX1). Tat<sub>47-57</sub>-Oct4 was secreted into the growth medium, yielding ~210 mg/l. The transmembrane transport ability of purified Tat<sub>47-57</sub>-Oct4 fusion protein was confirmed by immunofluorescence analysis.

## Materials and methods

**Acquisition of the Oct4 gene.** To obtain the Oct4 gene, total RNA was extracted from human livers and used as the template for reverse transcription, conducted for 30 min at 42°C followed by heat treatment for 2 min at 94°C to inactivate avian myeloblastosis virus reverse transcriptase. The cDNA was then used as the template for polymerase chain reaction (PCR) using the following primers: P1, 5'-CCATGGCGGGACACCTGGCTTC-3' and P2, 5'-TCAGTTTGAATGCATGGGTG-3'.

The PCR protocol consisted of initial pre-heating at 94°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec and a 5-min final elongation at 72°C. The PCR product was inserted into the PMD-18 vector to generate the plasmid, pTOct4, which was verified by DNA sequencing.

**Construction of expression vector, pPICZαC-Tat-Oct4.** Three primers were designed to construct a plasmid containing the human serum albumin (HSA) signal peptide and HIV Tat<sub>47-57</sub> fused with Oct4. Partial DNA sequences of the HSA signal peptide, HIV Tat<sub>47-57</sub> and a 13-bp homologue of the Oct4 DNA sequence were included in primer P3: Forward, 5'-TTATTTCGC GAGGTGTGTTTCGTCGATACGGTAGAAAGAAGCGTCCG ACAGCGTCGACGAATGGCGGGACACC-3'. The homologue DNA sequence of Oct4 is shown in bold, the DNA

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**Key words:** human Oct4, *Pichia pastoris*, fusion protein

sequence of HIV Tat<sub>47-57</sub> is underlined and the partial DNA sequence of the HSA signal peptide is shown in italics. The DNA sequences of the Bsp104I recognition sites and the HSA signal peptide were included in P4: Forward, 5'-GGTTC GAAACGATGAAGTGGGTAACCTTTATTTCCCTTCTTTTC TCTTTAGCTCGGCTTATTCGCGAGGTGTG-3'. The DNA sequence of the HSA signal peptide is in italics, and the DNA sequence of the recognition site for Bsp104I is underlined. A total of 17 bp of reverse homologue DNA sequences of Oct4 and the XbaI recognition sites were included in P5: Forward, 5'-CCAGAATTCTCAGTTTGAATGCATGG-3'. The DNA sequence of the recognition site for XbaI is underlined and the stop codon is shown in bold.

Two rounds of PCR were performed. Firstly, the plasmid pTOct4 was used as the template in the first PCR with primers P3 and P2. The PCR reaction mixture was initially heat denatured at 94°C for 5 min, followed by 16 cycles with 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C and 90 sec of extension at 72°C, 30 sec of denaturation at 94°C, 30 sec annealing at 53°C and 90 sec extension at 72°C. An additional extension for 5 min at 72°C was performed to ensure the completion of the PCR products. Secondly, the PCR products were used as the template for the second PCR with primers P4 and P5. The cycle program consisted of 33 cycles with 30 sec of denaturation at 94°C, 30 sec of annealing at 59°C and 90 sec of extension at 72°C and a final extension of 5 min at 72°C. The product was digested with Bsp104I and XbaI and ligated into pPICZαC at the same sites to generate pPICZαC-pHSA-Tat-Oct4. The result was confirmed by DNA sequencing.

**Screening for high-level expression colonies.** The recombinant expression vector, pPICZαC-pHSA-Tat-Oct4, was linearized by SacI and then introduced into *P. pastoris* X-33 (Invitrogen Life Technologies, Carlsbad, CA, USA) by electroporation with a Micropulser (Bio-Rad, Hercules, CA, USA) according to the pPICZαC vector manual. The transformants were screened on yeast extract peptone dextrose (YPD) agar plates containing zeocin, and cultured at 28°C for at least three days. The positive clones were selected and cultured in 5 ml buffered glycerol-complex (BMGY) medium at 28°C for 24 h with agitation at 250 rpm in an orbital shaker (Thermo Fisher Scientific, Boston, MA, USA). The genomic DNA was extracted and amplified using 5'AOX1 and 3'AOX1 as primers to verify whether the Tat-Oct4 gene was integrated into the genome stably. Non-transformed yeast DNA was extracted as a control group. To achieve a high yield of Tat-Oct4, the positive transformants were cultured in 10 ml BMGY medium [1.0% yeast extract, 2.0% peptone, 1.34% yeast nitrogen base, 0.5 mg/l biotin, 100 mM potassium phosphate (pH 6.0) and 1.0% glycerol] and incubated at 28°C for 24 h. Next, the cells were cultured in 10 ml buffered methanol-complex medium in which 1.0% glycerol was replaced by 0.5% methanol. Fresh methanol was added every 24 h to maintain the concentration at 0.5% (v/v) for nine days. The expression level of Tat-Oct4 in the supernatant was determined by SDS-PAGE and western blotting.

**Pilot-scale fermentation of Tat<sub>47-57</sub>-Oct4.** The clone with the highest level of Tat-Oct4 expression was cultured in 2 liters YPD medium in a 5-liter conical flask in a shaking incubator (Thermo Fisher Scientific), at 28°C until the optical density

(OD) of cultured *P. pastoris* at 600 nm reached 10. This culture was then added into a 80-liter NBS Bioflo 5000 fermenter (New Brunswick Scientific, Enfield, CT, USA) containing 40 liters of fermentation basal salt medium FM21, supplemented with PTM1 trace salts (21) and biotin (0.04 ml stock solution). The level of dissolved oxygen (DO) was maintained at 30-40% and the stirring rate was 400 rpm in the 80-liter NBS Bioflo 5000 fermenter (New Brunswick Scientific, Enfield, CT, USA). The medium was maintained at pH 4.0 by automatic addition of 5M NH<sub>4</sub>OH and 1M phosphoric acid and 5% antifoam was also delivered as required. The temperature was controlled at 28°C. The fermentation was divided into three phases; the designated glycerol, glycerol-fed and methanol-fed batch phases. The pH of the medium was maintained at 4.0 during the glycerol phase and once the glycerol was consumed at the end of the first phase, there was a sharp increase in DO value, and the second phase was initiated. During this phase, 50% glycerol feed, containing 1.2% (v/v) PTM1 trace salts, was added at an initial speed of 400 ml/h with peristaltic pump (LongerPump, Baoding Hebei, China), with the speed of addition gradually increasing to 720 ml/h. The glycerol was supplied until a cell yield of 180-220 g/l wet weight was achieved. The third phase was initiated by starting a 100% methanol feed containing 1.2% (v/v) PTM1 trace salts. During the methanol induction phase, the pH was adjusted to 8.0. Methanol was initially added at 144 ml/h for 4 h to allow the culture to adapt to growth on methanol, subsequent to which, the addition speed was gradually increased to 440 ml/h. The samples of the culture medium were collected every 4 h to analyze the wet cell weight, the OD<sub>600</sub> and the expression level of Tat-Oct4.

**Purification of Tat<sub>47-57</sub>-Oct4.** The supernatant was harvested by centrifugation (10,000 x g, 5 min) and the proteins were precipitated by 35% saturated ammonium sulfate. As the molecular weight of Tat<sub>47-57</sub>-Oct4 is ~40 kDa, all the proteins weighing between 10 and 100 kDa were isolated and concentrated using Vivaflow 200 PES with 50,000 and 10,000 molecular weight cut off (Sartorius, Goettingen, Germany). To purify the sample further, the supernatant was diluted three times with 50 mM NaAc-HAc and loaded onto a SP Sepharose column (Amersham-Pharmacia, Piscataway, NJ, USA), pre-equilibrated with 50 mM NaAc-HAc buffer and set at a flow rate of 30 ml/min. The protein was eluted in a linear salt gradient and monitored by measuring the UV absorbance at 280 nm. The fractions containing Tat-Oct4 (from the SP Sepharose XL column) were desalted and concentrated by ultrafiltration (10,000 molecular weight cut off, Vivaflow 200) and filtered by a 0.22-μm filter. The components were analyzed by SDS-PAGE and western blotting to determine which contained Tat-Oct4. The purified Tat-Oct4 was analyzed on a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) using a C4 reversed-phase column (Waters, Milford, MA, USA). The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the concentration standard (22). The purified Tat-Oct4 was freeze-dried rapidly in a high vacuum freeze dryer, ALPHA-1-4 (Martin Christ Company, Harz, Germany). The concentration of Tat-Oct4 at each step of the procedure was quantified by an enzyme-linked immunosorbent assay and the product was stored under sterile conditions at -80°C.

**SDS-PAGE and western blotting.** The purified protein, Tat-Oct4 was analyzed by SDS-PAGE performed with a 12% gel and stained with Coomassie brilliant blue, according to the method of Sambrook and Russel (23).

The proteins in the gel were transferred to a polyvinylidene fluoride (PVDF) membrane for western blotting using a semi-dry electroblotting apparatus (Bio-Rad) at 15 V for 30 min in 25 mM Tris/192 mM glycine. The membrane was blocked by incubating with Tris-buffered saline with Tween 20 (TBST) containing 2% BSA over 12 h at 4°C, then washed three times with 0.2% BSA in TBST and incubated with rabbit anti-human Oct4 polyclonal antibody (Abcam, Cambridge, UK) for 3 h at room temperature, followed by a final three washes with TBST. The membrane was then incubated with the secondary goat anti-rabbit antibody (Abcam, Boston, MA, USA) for another 3 h, washed three times with TBST and then washed with TBS for 15 min. The Tat-Oct4 fusion protein was detected using 3,3'-diaminobenzidine tetrahydrochloride reagents (Beyotime, Jiangsu, China).

**N-terminal amino acid sequence analysis.** To determine the N-terminal sequence, the purified Tat-Oct4 was electrophoresed on a 12% SDS-PAGE gel and electroblotted onto a PVDF membrane. Following blotting, the PVDF membrane was stained with Amido black and the Tat-Oct4 band was cut out. The N-terminal amino acid sequence analysis was conducted using a PPSQ-21A protein sequencer (Shimadzu, Kyoto, Japan).

**Detection of transmembrane transport ability of Tat-Oct4.** Fibroblasts were obtained from human foreskin tissue, obtained from the first hospital of Jilin University and the patient consent was signed by the patient himself. The fibroblasts were inoculated in a 24-well plate at a density of  $1 \times 10^5$  cells per well. After 24 h, the cells were treated with serum-free minimum essential medium containing 1  $\mu$ M Tat-Oct4 or saline water (negative control group), and cultured with serum-free medium for 6 h only. The cells were then washed three times with phosphate-buffered saline (PBS) for 10 min to remove proteins from the outside of the cells, fixed with 4% paraformaldehyde for 30 min, washed three times with PBS for 5 min and then treated with 0.25% Triton X-100 in PBS and 5% BSA (to block non-specific binding) for 30 min. The cells were then incubated overnight at 4°C with rabbit Oct4 polyclonal antibody (Abcam, Cambridge, UK), then washed three times with PBS for 5 min and incubated further with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody for 30 min at 37°C. The cells were rinsed three times and incubated with propidium iodide solution. Finally, the cells were rinsed another three times and examined using a fluorescence microscope (FA500; Olympus, Tokyo, Japan).

## Results and Discussion

**Construction and transformation of Tat-Oct4.** The 1,086-bp gene fragment encoding human Oct4 was amplified by PCR with primers P1 and P2, and subcloned into the PMD-18 vector to generate the plasmid, pTOct4. The 1,200-bp gene fragment encoding the HSA signal peptide, HIV Tat<sub>47-57</sub>, Oct4 Bspt1041 and the *Xba*I recognition site, was amplified by two-step PCR from pTOct4 and subcloned into pPICZ $\alpha$ C to generate the recombinant expression vector, pPICZ $\alpha$ C-Tat-Oct4.

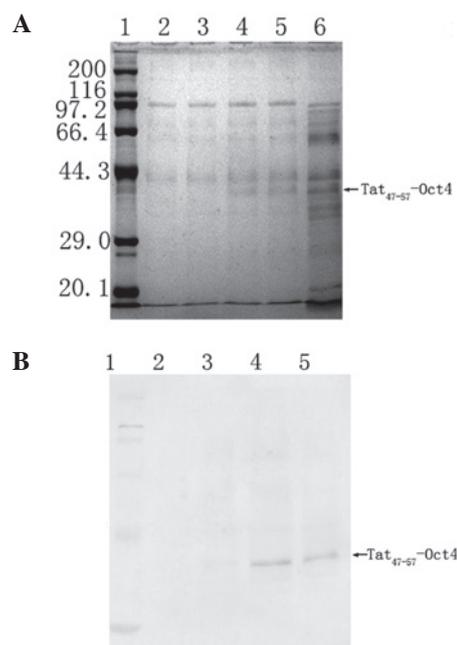


Figure 1. (A) Coomassie-stained SDS-PAGE profile of *Pichia pastoris* clones expressing Tat-Oct4. Lane 1, broad molecular weight protein marker (6.5-200 kDa); lane 2, supernatant of *P. pastoris* X33; lane 3, supernatant of the *P. pastoris* strain transformed with pPICZ $\alpha$ C; and lanes 4-6, supernatant of the *P. pastoris* strain transformed with pPICZ $\alpha$ C-Tat-Oct4. (B) Western blot of recombinant *Pichia* supernatant expressing Tat-Oct4. Lane 1, broad molecular weight protein marker (6.5-200 kDa); lane 2, supernatant of *P. pastoris* X33; lane 3, supernatant of the *P. pastoris* strain transformed with pPICZ $\alpha$ C; and lanes 4 and 5, supernatant of the *P. pastoris* strain transformed with pPICZ $\alpha$ C-Tat-Oct4.

Nucleotide sequencing analysis confirmed that the two vectors contained the correct gene.

**Expression of *P. pastoris* and screening for high-level expression colonies.** To integrate the HSA signal peptide, HIV Tat<sub>47-57</sub> and Oct4 were incorporated into the genome of *P. pastoris* by homologous recombination. pPICZ $\alpha$ C-Tat-Oct4 was linearized with *Sac*I and transformed into electrocompetent *P. pastoris* cells. A successful integration was confirmed by screening with zeocin and PCR with AOX1 universal primers. The PCR results revealed that 95% zeocin-positive yeast transformants had one specific band of ~1,471 bp.

The positive clones were inoculated in BMGY medium for 24 h, the cells were collected and the expression of the Tat<sub>47-57</sub>-Oct4 fusion protein was induced in the buffered methanol-complex medium. Subsequent to a 168-h induction, each strain was analyzed on SDS-PAGE to detect the Tat<sub>47-57</sub>-Oct4 expression. According to the DNA sequence of Tat-Oct4, the calculated molecular weight of Tat-Oct4 was ~40 kDa (Fig. 1).

**Pilot-scale expression, purification and characterization of purified Tat<sub>47-57</sub>-Oct4.** All the details of the batch fermentation are shown in Fig. 2. The glycerol phase lasted 30 h and the methanol induction phase lasted 112 h. At the end of the glycerol phase, the OD<sub>600</sub> of the culture reached 168; the wet cell weight reached 221 mg/ml and the DO value sharply increased from 30 to 100 mg/ml. In order to consume all the glycerol, the cells were cultured for an additional 2 h without any additions. Methanol was then added into the culture as the inducer and the carbon source during *P. pastoris* fermentation. The methanol feed rate

Table I. Summary of purification steps of Tat<sub>47-57</sub>-Oct4 from *P. pastoris*.

Purification steps	Total proteins, mg	Tat <sub>47-57</sub> -Oct4, mg	Recovery, %	Purity, %
Supernatant	252900	15100		4.98
Vivaflow 200	87100	12080	80.50	13.86
SP Sepharose XL	7510	6864	56.00	91.34
Vivaspin	6010	5750	85.00	95.60

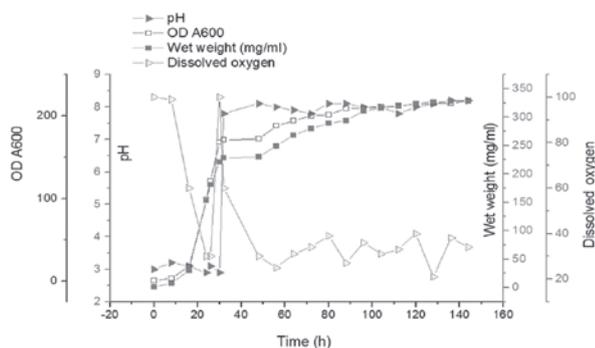
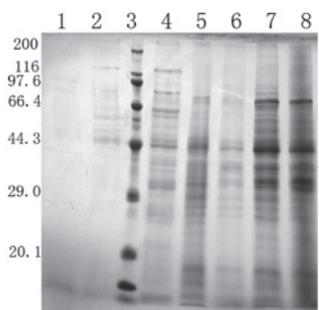


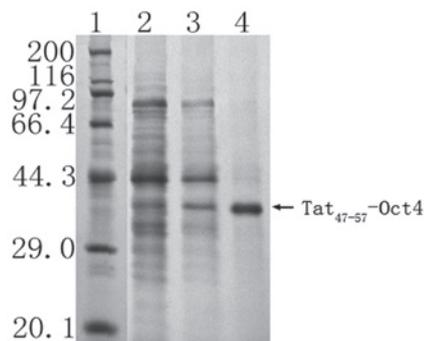
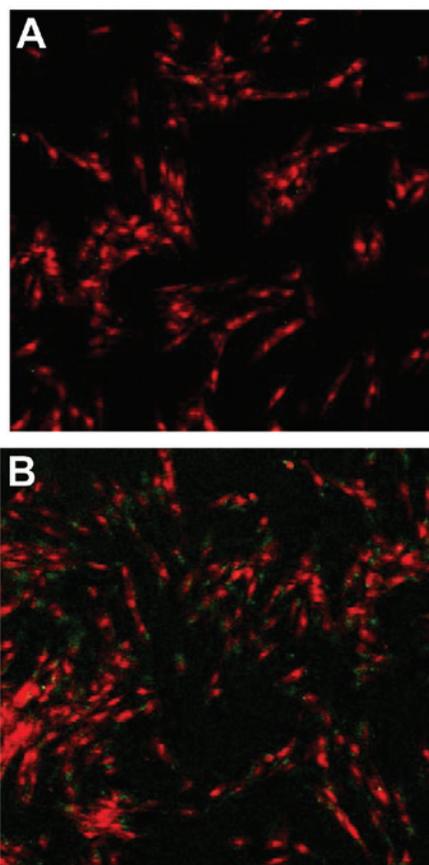
Figure 2. Graph to show the individual parameters during the batch fermentation process of Oct4 production by an automatic fermenter. OD A600, optical density absorbance at 600nm.

Figure 3. Coomassie-stained SDS-PAGE profile of the expression levels of Tat<sub>47-57</sub>-Oct4 during fermentation. Lane 3, broad molecular weight protein marker (6.5-200 kDa); and lanes 1, 2 and 4-8, supernatant samples showing the expression levels during fermentation of Tat<sub>47-57</sub>-Oct4 at 0, 24 (glycerol phase; no band at 40 kDa), 48, 72, 96, 120 and 144 h [methanol-fed phase; showing one specific band ~40 kDa (between 29 and 44 kDa) indicated by the arrow].

was adapted according to the DO value, which was maintained at 30-40%. The weight of wet cells and the corresponding OD<sub>600</sub> reached 328 and 218 mg/ml subsequent to a 112-h induction. The expression level of Tat<sub>47-57</sub>-Oct4 during fermentation was revealed by Coomassie-stained SDS-PAGE (Fig. 3).

**Purity and yield of Tat<sub>47-57</sub>-Oct4.** Following the fermentation and purification processes, 601 mg purified Tat<sub>47-57</sub>-Oct4 was obtained from 60 liters of culture medium. The Tat<sub>47-57</sub>-Oct4 purity was 95.6%, as revealed by SDS-PAGE (Fig. 4) and HPLC. The protein recovery ratio and purity of Tat<sub>47-57</sub>-Oct4 at the various purification steps are summarized in Table I.

**N-terminal amino acid sequence analysis.** The signal peptide and propeptide of HSA, which consists of a signal sequence of 24 amino acids (MKWVTFISLLFLFSSAYSRGVFR),

Figure 4. SDS-PAGE analysis of the purified Tat<sub>47-57</sub>-Oct4. Lane 1, broad molecular weight protein marker (6.5-200 kDa); lane 2, supernatant of Tat<sub>47-57</sub>-Oct4; lane 3, Tat<sub>47-57</sub>-Oct4 purified with Vivaflow 200; and lane 4, Tat<sub>47-57</sub>-Oct4 purified with cation exchange chromatography and Vivaflow 200.Figure 5. Immunofluorescence analysis of the ability of Tat<sub>47-57</sub>-Oct4 to cross the cell membrane. (A) Saline, negative control; and (B) Tat<sub>47-57</sub>-Oct4 group. The chromatin stained with propidium iodide emits a red fluorescence. The Tat-Oct4, which is indirectly labeled by the fluorescein isothiocyanate-labeled-conjugated antibody, emits a green fluorescence. Magnification, x100.

can be cleaved at the site after amino acid residues SR or RR. The N-terminal sequencing of the purified protein revealed the sequence of the first 15 amino acids as GVFRRYGRKKRRQR, indicating that the signal peptide was cleaved at the site after amino acid residue SR.

#### Detection of the Tat-Oct4 transmembrane transport ability.

The transmembrane transport ability of Tat-Oct4 was analyzed by indirect immunofluorescence. The results demonstrated that visible green fluorescence signals appeared in the human foreskin fibroblasts treated with serum-free medium containing Tat-Oct4. The green fluorescence signal was absent in the cells treated with saline (the negative control group), indicating that Tat<sub>47-57</sub>-Oct4 has the ability to pass through the cell membrane (Fig. 5).

Kim *et al* expressed Tat-SOD in *E.coli* and tested the protective effect against ischemic brain injury. They observed that Tat-SOD was able to enter brain neurons and protect them from ischemic insult and cell death (24). Liu *et al* used EPO and EPO-Tat to treat a rat model of transient focal ischemia and demonstrated that 1,000 U/kg EPO-TAT exhibited a comparable neuroprotection to 5,000 U/kg EPO with no detectable side effects (25). Furthermore Zhou *et al* used *E.coli* to produce human Oct4-11R-His fusion protein and observed the membrane penetrating ability of the fusion protein. They showed that the fusion protein was able to effectively enter the BJ cells and locate around the nuclei, but the authors did not investigate the bioactivity of the fusion protein (26). In the present study, the Tat<sub>47-58</sub>-Oct4 fusion protein was successfully expressed using the *P. pastoris* expression system and purified following pilot-scale fermentation by precipitation, ultrafiltration and chromatography. The yield and purity of Tat<sub>47-57</sub>-Oct4 were 210 mg/l and 95.6%, respectively, and the fusion protein revealed the ability to penetrate human foreskin fibroblasts. Therefore, the yeast expression system described in the present study is a useful tool to produce a large quantity of active Tat<sub>47-57</sub>-Oct4 fusion protein, which is likely to facilitate the mechanistic and potential clinical application studies of Oct4. In the next step we will investigate the bioactivity of the Tat<sub>47-57</sub>-Oct4 fusion protein in induced pluripotent stem (iPS) cells.

#### Acknowledgements

This study was supported by the Jilin Kangrui Regenerative Medicine Co., Ltd. Changchun, P.R. China.

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